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Virus-Specific CD8 T Cells in Peripheral Tissues Are More Resistant to Apoptosis Than Those in Lymphoid Organs

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Summary

CD8 T cells persist at high frequencies in peripheral organs after resolution of an immune response, and their presence in the periphery is important for resistance to secondary challenge. We show here that LCMV-specific T cells in peripheral tissue (peritoneal cavity, lung, fat pads) reacted much less with the apoptotic marker Annexin-V than those in spleen and lymph nodes. This was not due to a TCR-based selection. In comparison to lymphoid tissue, T cells in the periphery expressed lower levels of Fas and Fas ligand and were resistant to activation-induced cell death in vitro. This may contribute to the survival of nondividing peripheral memory T cells, enabling them to efficiently function without being driven into apoptosis.

Introduction

Vigorous CD8 T cell responses that occur during viral infections usually come to a halt shortly after infectious virus is cleared, and homeostasis is restored by dramatic reductions in their numbers in the spleen and lymph nodes (Ahmed and Gray, 1996; Razvi et al., 1995a). Many of these CD8 T cells undergo apoptosis (Razvi et al., 1995a), whereas a few are spared death and enter the long-lasting memory pool (Ahmed and Gray, 1996; Zinkernagel et al., 1996). Other T cells disperse into peripheral organs, where they reside at relatively high frequencies as peripheral memory cells, ready to respond to secondary stimulation on re-encounter with the pathogen (Chen et al., 2001; Hogan et al., 2001; Mackay et al., 1992; Marshall et al., 2001; Masopust et al., 2001; Reinhardt et al., 2001).

Highly activated CD8 T cells are prone to undergo apoptosis, in part because they express low levels of antiapoptotic proteins of the Bcl-2 family (Broome et al., 1995). They also may express Fas and Fas ligand and TNF and TNF receptor, and, if stimulated strongly through their T cell receptor (TCR), they undergo apoptosis by the mechanism of activation-induced cell death (AICD) (Lenardo et al., 1999; Russell et al., 1991). Triggering through the TCR can, in fact, rapidly upregulate Fas and FasL expression in T cells that have been previously activated (Lenardo et al., 1999; Nagata and Golstein, 1995). AICD may not, however, be the mechanism for the silencing of the T cell response at the termination of infection. Antigen is no longer present to trigger the TCR, and CD8 T cells from mice deficient in AICD cofactors such as Fas, FasL, and TNF undergo silencing normally (Lohman et al., 1996; Razvi et al., 1995a; Reich et al., 2000; Zimmermann et al., 1996b). This death might be a consequence of growth factor removal (Deng and Podack, 1993) or even may be a preprogrammed response to occur after a resting T cell has undergone a specific number of cell divisions. The silencing of the T cell response is not blocked in mice whose T cells express a Bcl-2 transgene (Petschner et al., 1998; Razvi et al., 1995a), but surviving activated T cells that become memory cells express more Bcl-2 than the overall activated T cell population (Grayson et al., 2000).

Memory CD8 T cells are heterogeneous in antigenic phenotype and phase of cell cycle (Mullbacher and Flynn, 1996; Razvi et al., 1995b; Tough and Sprent, 1994). It has been suggested that there may be at least two kinds of memory CD8 T cells—"central" memory cells that are resting cytolytically inactive cells that can proliferate in response to antigen and rapidly replenish the effector cell pool, and "effector" memory cells that are more terminally differentiated in regards to heightened levels of cytolytic effector function and more limited cell division potential (Masopust et al., 2001; Reinhardt et al., 2001; Sallusto et al., 1999). The effector memory cells may be at increased frequency in peripheral tissue, where they would serve to engage a pathogen on secondary infection.

What has not been analyzed is whether the CD8 T cell apoptosis that terminates an immune response occurs equally in all organs. Differential sensitivity to apoptosis at the termination of an immune response could lead to a disproportionate skewing of memory T cells in some organs but not others. Resistance to apoptosis could allow for the survival of a long-lasting effector T cell population that could productively respond to antigen rechallenge without undergoing AICD. Here, we provide evidence in mice acutely infected with lymphocytic choriomeningitis virus (LCMV) that antigen-specific CD8 T cells from the lymphoid organs and the peripheral tissue differ dramatically in apoptotic markers and in their susceptibility to FasL-mediated AICD.

Results

Reactivity of MHC Dimer-Identified Antigen-Specific CD8 T Cells with Annexin-V

Among the many assays for the detection of apoptotic cells, few have been useful for quantifying dying cells in vivo because macrophages phagocytize apoptotic cells via scavenging receptors for phosphatidyl serine, which is expressed very early on the surface of apoptotic cells (van Engeland et al., 1998). Expression of phosphatidyl serine, which can be detected by Annexin-V, occurs prior to events associated with DNA degradation, complete caspase pathway activation, and alterations in membrane permeability. We therefore used Annexin-V to identify putatively preapoptotic CD8 T cells in the lymphoid and peripheral organs of mice infected with LCMV, and for these experiments we identified the antigen-specific T cells by costaining them with LCMV pep-

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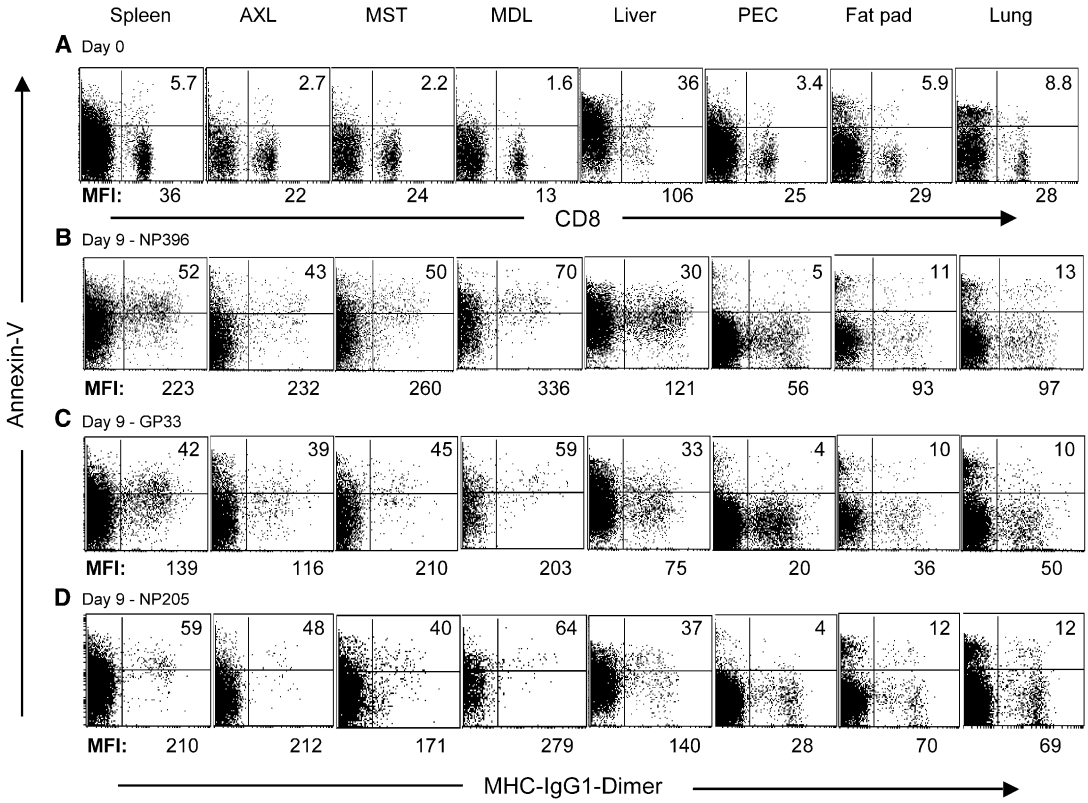

Figure 1. Low Frequency of Annexin-V Binding LCMV-Specific T Cells in Nonlymphoid Organs

Leukocytes were isolated from day 0 (A) or day 9 LCMV acutely infected C57BL/6 mice and stained with NP396 (B)-, GP33 (C)-, or NP205 (D)-specific IgG1-MHC dimers, anti-CD8, and Annexin-V. Data were gated on lymphocyte (A) or CD8 cells (B–D) with 300–700 forward scatter (FSC) units. The organs were spleen, axillary lymph node (AXL), mesenteric lymph node (MST), mediastinal lymph node (MDL), liver, peritoneal cavity (PEC), fat pad, and lung. The *y* axis represents Annexin-V staining, and the *x* axis represents CD8 (A) or dimer (B–D) staining. The numbers recorded in the right upper quadrants represent the percent of CD8 (A)- or LCMV-specific CD8 T cells (B–D) staining with Annexin-V. The numbers under each panel represent the mean fluorescent intensity (MFI) of Annexin-V staining of CD8 (A)- or LCMV-specific CD8 T cells (B–D). All the day 9 data were derived from one single mouse, but they were representative of six individual experiments with two to three mice in each experiment.

tide-charged MHC-dimers. Staining was done in the cold to prevent ligand-induced AICD of the T cells.

Figure 1 shows Annexin-V binding of NP396-, GP33-, and NP205-specific CD8 T cells in various organs of mice 9 days after intraperitoneal LCMV infection. The results show, for each LCMV epitope, a much higher reactivity with Annexin-V in the spleen and lymph nodes than in several peripheral organs, including the peritoneal cavity, lung, and fat pad. These differences could be shown by scoring the frequency of Annexin-V "+" cells, depicted in the upper right hand quadrants, and by the less arbitrary mean fluorescent intensities (MFI) within the antigen-specific T cell populations (Figure 1). The liver, previously described to be a graveyard for apoptotic CD8 T cells in other systems (Huang et al., 1994; Liu et al., 2001), had intermediate levels of Annexin-V staining. Annexin-V staining in every tissue was higher in LCMV-specific cells than in the overall CD8 T cell population (Table 1), and, except in the liver, Annexin-V staining of CD8 T cells prior to infection was quite low. Table 1 shows additional data for days preceding or following the day 9 peak in the T cell response, and the same pattern was seen for each time point. Of note, and for unknown reasons, higher proportions of Annexin-V binding cells were seen in T cells of some specificities (e.g., NP396 and NP205) than of other specificities (e.g., GP33), but T cells of each specificity demonstrated similar organ-dependent differences. Table 1 also shows data compiled from mice 2 and 4 months after resolution of the LCMV infection to determine whether the tissue-dependent differences were maintained in the memory state. Surprisingly, there was still substantially more Annexin-V binding in antigen-specific T cells in the spleen and lymph nodes than in the peritoneal cavity, fat pads, or lungs. Thus, the phenotype is long-lasting. The data presented in Figure 1 and Table 1 were based on "viable" lymphocyte gates, spanning 300–700 forward scatter (FSC) units, but it should be noted that the dramatic tissue-related differences in Annexin-V binding T cells were still apparent even when "wide-open" gates were examined (e.g., for the NP396$^+$ cells in Figure 1B gated at FSC 200–800, spleen = 59%; PEC = 7.7%).

The silencing of the LCMV-induced T cell response occurs independently of Fas and FasL expression (Lohman et al., 1996; Razvi et al., 1995a; Zimmermann et al., 1996b). Consistent with this observation, antigen-specific T cells from LCMV-infected lpr and gld mice

Table 1. Percentage of Annexin-V-Positive Antigen-Specific $CD8^+$ T Cells in Lymphoid and Nonlymphoid Organs during Acute LCMV Infection

		Annexin-V^+ (%)							
		Spleen	AXL	MST	MDL	Liver	PEC	Fat Pad	Lung
Day 0	$CD8^+$	4.7 ± 1.4	2.2 ± 0.7	2.6 ± 0.5	2.2 ± 0.9	32 ± 6.7	2.6 ± 1.2	5.2 ± 0.9	8.9 ± 0.1
Day 7 p.i.	$CD8^+$	38 ± 4.7	11 ± 2.9	17 ± 2.3	27 ± 3.4	34 ± 12	7.2 ± 6.1	9.2 ± 0.9	22 ± 5.1
	$NP396^+$	70 ± 3.0	34 ± 16	53 ± 2.0	51 ± 6.9	48 ± 3.3	7.6 ± 3.5	11 ± 3.2	24 ± 7.3
	$GP33^+$	51 ± 14	25 ± 14	43 ± 3.2	37 ± 9.6	39 ± 7.6	5.4 ± 0.8	9.1 ± 1.9	19 ± 6.7
	$NP205^+$	62 ± 23	38 ± 7.6	54 ± 1.0	45 ± 2.5	44 ± 1.2	6.7 ± 1.9	1.4 ± 4.6	18 ± 4.4
Day 9 p.i.	$CD8^+$	26 ± 4.1	12 ± 3.0	19 ± 5.4	26 ± 9.6	30 ± 5.8	6.3 ± 4.2	7.2 ± 2.2	10 ± 3.4
	$NP396^+$	44 ± 9.3	36 ± 11	49 ± 11	62 ± 12	32 ± 5.4	6.7 ± 5.4	7.1 ± 1.5	8.9 ± 1.9
	$GP33^+$	38 ± 5.4	26 ± 10	35 ± 15	37 ± 18	21 ± 0.4	2.7 ± 0.9	4.6 ± 1.6	7.0 ± 1.9
	$NP205^+$	47 ± 11	34 ± 12	45 ± 13	62 ± 14	36 ± 2.9	5.3 ± 2.1	8.2 ± 4.3	8.8 ± 4.5
Day 12 p.i.	$CD8^+$	34 ± 6.8	6.7 ± 3.1	15 ± 5.2	17 ± 1.2	18 ± 7.6	3.4 ± 1.4	2.9 ± 0.3	7.4 ± 2.3
	$NP396^+$	57 ± 9.4	34 ± 12	57 ± 4.2	62 ± 8.5	28 ± 7.7	4.2 ± 1.9	5.1 ± 1.3	7.6 ± 0.4
	$GP33^+$	50 ± 8.1	22 ± 0.5	39 ± 0.1	42 ± 0.8	23 ± 8.7	3.1 ± 0.1	3.0 ± 1.0	7.3 ± 0.5
	$NP205^+$	51 ± 9.6	33 ± 3.1	53 ± 5.2	53 ± 0.2	34 ± 1.2	4.5 ± 0.5	5.8 ± 0.4	10 ± 2.3
2–4[a] months p.i.	$CD8^+$	8.4 ± 0.9	4.3 ± 1.2	5.5 ± 0.5	6.2 ± 1.6	16 ± 1.4	6.4 ± 3.6	4.8 ± 2.5	3.8 ± 1.8
	$NP396^+$	47 ± 5.6	37 ± 6.7	47 ± 11	31 ± 15	25 ± 4.0	15 ± 5.8	13 ± 4.8	5.4 ± 0.6
	$GP33^+$	28 ± 2.2	14 ± 9.0	30 ± 22	11 ± 2.1	23 ± 1.7	11 ± 5.5	14 ± 4.7	5.5 ± 2.4

The % of Annexin-V^+ total $CD8^+$ cells on LCMV-epitope-specific $CD8^+$ T cells was determined at different days postinfection, as described in the legend to Figure 1. (N = 3 to six individuals)

[a] Data average from two mice 2 months p.i. and two mice 4 months p.i.

had similar Annexin-V reactivity as those in wild-type mice (e.g., % Annexin-V staining of $NP396^+$ cells at day 9 p.i. in the spleen, AXL, PEC, and fat pad: gld-42, 46, 2.4, 5.3; lpr-40, 57, 3.9, 6.9).

No Evidence for TCR-Based Affinity Maturation

During infections, there is an antigen-driven selection for T cells based on their affinity or avidity with MHC-bearing peptide ligands (Chen et al., 2000). We therefore questioned whether the lack of Annexin-V reactivity of T cells in peripheral organs was a consequence of a TCR-based selection of T cells. CDR3 spectratype analyses were performed on antigen-specific T cells that had been stained with NP396 peptide-charged MHC-tetramers, sorted by FACS, and analyzed for the existence of dominant $V\beta8.1^+$ clones in windows defined by Jβ1.1, 1.2, 1.3, 1.4, 1.5, and 2.1. Figure 2 shows results from three experiments. Similar spectratypes of NP396-specific T cells purified from spleen, PEC, fat pad, and lung (day 9 postinfection) are shown for 6 Jβ windows in Figure 2A. A total of five experiments showed that the antigen-specific spectratypes were similar from organ to organ within individual mice. In the two experiments shown in Figures 2B and 2C, dominant singular Jβ1.1 and 1.2 peaks of sorted NP396-specific cells from spleen, fat pad, and lung of individual mice were sequenced across the CDR3 region. Identical dominant sequences were seen among the different organs, but the sequences of the two mice differed from each other, as would be predicted (Lin and Welsh, 1998). These experiments argue against the concept of a TCR-based selection of T cells in the peripheral organs being responsible for the differences in Annexin-V staining.

To further establish that the organ-dependent differences in Annexin-V-reactive T cells were not functions of T cell selection, studies were performed on LCMV GP33-specific transgenic Thy 1.2^+ T cells that were inoculated into Thy 1.1^+ congenic mice and traced during an LCMV infection. LCMV induces a dramatic expansion of these cells, which then undergo apoptosis (Petschner et al., 1998; Zimmermann et al., 1996a) and react with Annexin-V (Figure 3). The GP33-transgenic T cells stained with Annexin-V at relatively high levels in the spleen and axillary lymph node (similar data from mesenteric lymph node are not shown), but hardly at all in the peritoneal cavity, fat pads, and lung. This clearly established that this organ-dependent variance in Annexin-V staining was not TCR based, and it provided a mechanism to examine a TCR homogeneous population of T cells in different organs.

Bona Fide Apoptosis versus Delayed Clearance

We were surprised at the high frequencies of Annexin-V binding cells in the lymphoid organs and questioned if Annexin-V was detecting a stage in cell activation unrelated to apoptosis. However, immunofluorescent staining of known T cell activation markers including 1B11(CD43), CD44, CD69, CD122, and Ly6C showed as high or higher expression of these antigens on peripheral T cells (data not shown). This high frequency of Annexin-V^+ T cells in lymphoid tissue might reflect a delayed clearance of apoptotic cells in lymphoid tissue compared to peripheral tissue. It should be noted, however, that the TUNEL assay, which measures cells in later stages of apoptosis undergoing DNA fragmentation, detected very few ($<$6%) antigen-specific T cells in the spleen (gated as above), suggesting that the spleen clearance pathway was functioning effectively.

The TUNEL assay is not useful for quantifying apoptosis in lymphocytes immediately ex vivo but can be reliable once cells are put into culture. We therefore questioned whether Annexin-V^+ splenic T cells were committed to undergo DNA fragmentation by FACS-sorting Annexin-V^+ from Annexin-V^- splenocytes, putting them into culture for 5 hr at 37°C and then staining them with the TUNEL assay to monitor DNA fragmentation. Here, day 9 LCMV-infected Thy1.1^+ mice implanted with Thy1.2^+ LCMV-specific transgenic T cells were used, and trans-

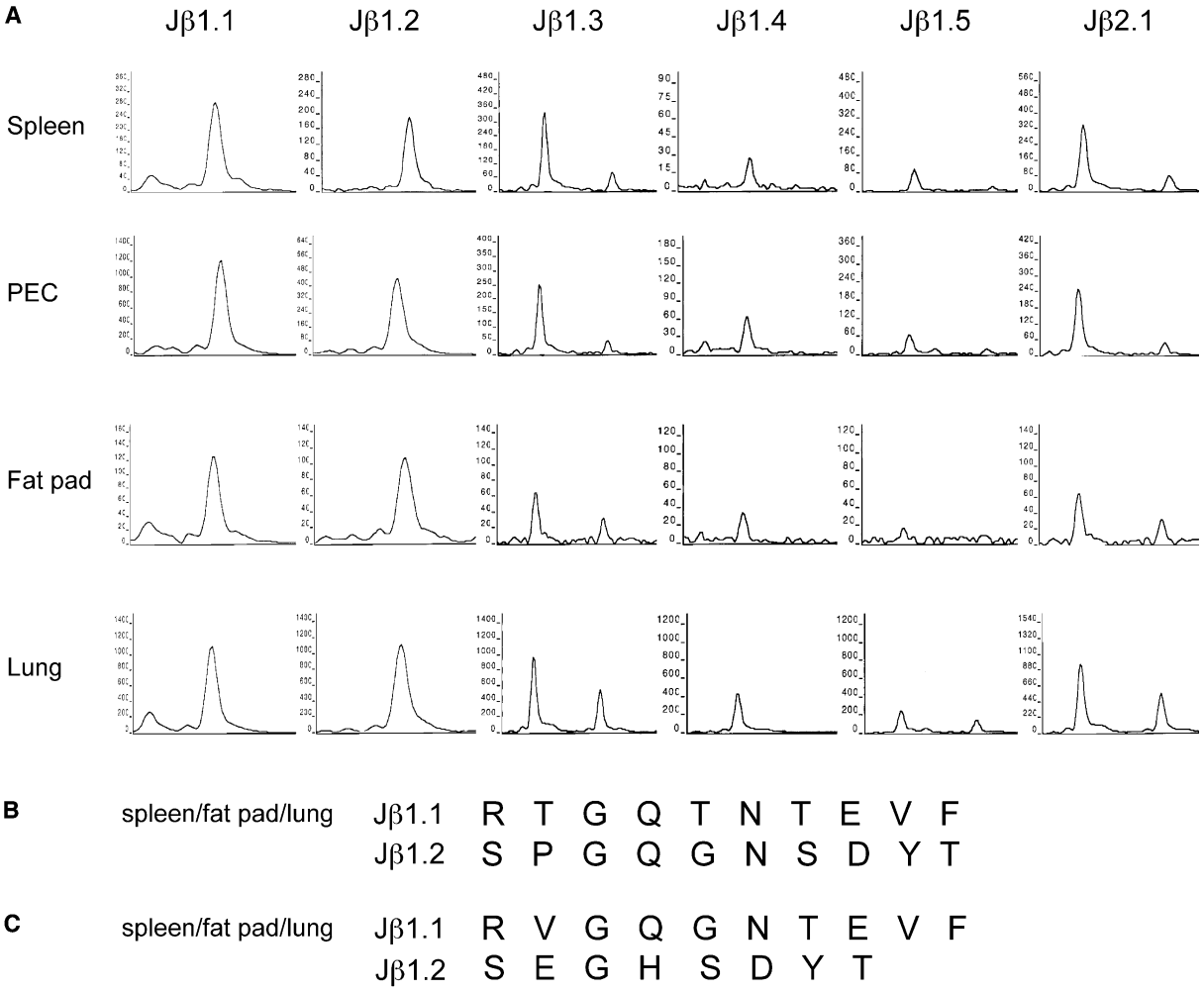

Figure 2. Similar Vβ8.1 CDR3-Length Spectratypes of CD8$^+$NP396 Tetramer$^+$ Cells from Different Organs of a LCMV Day 9 Infected Mouse

(A) Spectratypes of 6 Jβs from four organs of an individual mouse. Fluorescent intensity (*y* axis) was plotted against relative CDR3 size (*x* axis). (B and C) Sequences of CDR3 regions of dominant Jβ1.1 and 1.2 peaks from three organs of two individual mice.

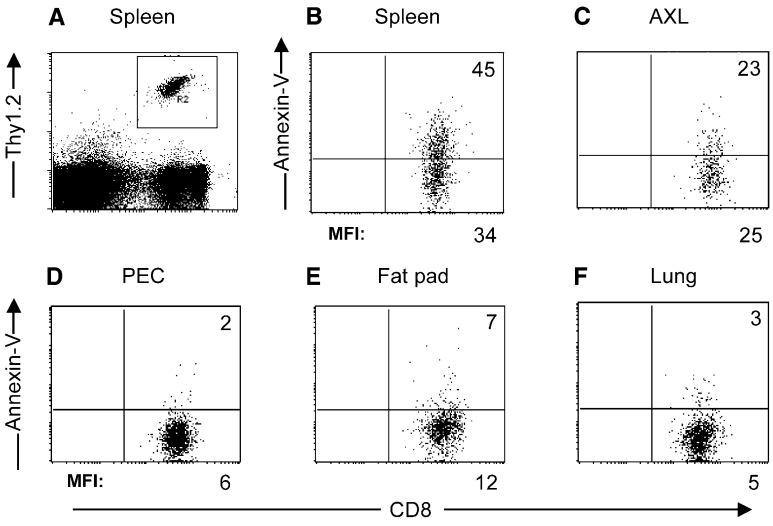

Figure 3. Low Frequency of Annexin-V Binding LCMV-Specific Transgenic T Cells in Non-lymphoid Organs

Thy1.2$^+$ LCMV-specific transgenic T cells were adoptively transferred into Thy1.1$^+$ B6 mice. Nine days after LCMV infection, leukocytes were stained with anti-Thy1.2, anti-CD8, and Annexin-V. Data were gated on CD8$^+$Thy1.2$^+$ transgenic cells as shown in (A) for a spleen sample. The Annexin-V versus CD8 stainings of donor cells from spleen (B), AXL (C), PEC (D), fat pad (E), and lung (F) are shown. The numbers in the right upper quadrants represent the percent of transgenic T cells staining as Annexin-V$^+$. The numbers under each panel represent the MFI of Annexin-V staining of the transgenic T cells. Data were from one single mouse and were representative of five individual experiments with two to three mice in each experiment.

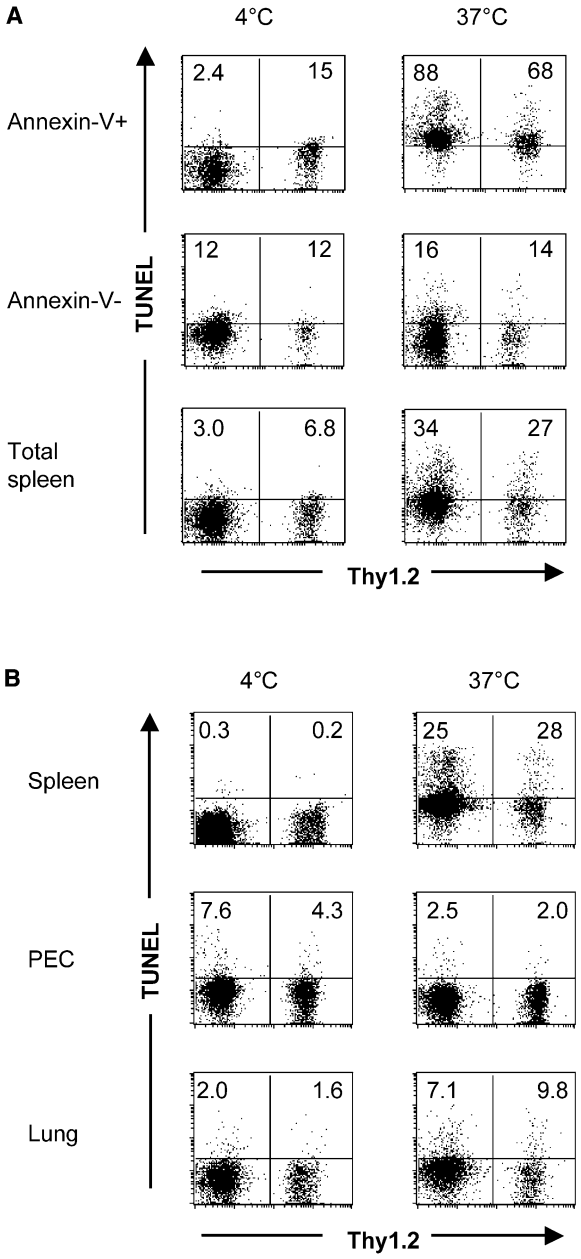

Figure 4. TUNEL Staining of Freshly Isolated T Cells after Short In Vitro Incubation

(A) Splenocytes of Thy1.1 mice reconstituted with Thy1.2 LCMV transgenic T cells were stained with Annexin-V 9 days after infection. Annexin-V$^+$ and Annexin-V$^-$ cells were sorted and cultured at 37°C or 4°C (without IL-2) as control for 5 hr and then analyzed with TUNEL assay. "Total spleen" was not stained with Annexin-V or sorted.

(B) Unsorted leukocytes from spleen, PEC, and lung from similar mice were cultured at 37°C or 4°C for 5 hr and stained with TUNEL. Data were gated on CD8$^+$ cells. The numbers in the left upper and right upper quadrants represent the percentage of TUNEL staining of host CD8 T cells and of the transgenic T cells, respectively.

genic and host T cells were monitored on gated CD8$^+$ lymphocytes. Figure 4A shows that incubation at 37°C resulted in very high TUNEL staining of both transgenic and host T cells derived from the Annexin-V$^+$ population but not from the Annexin-V$^-$ population. This does not prove that all of the Annexin-V$^+$ cells would have fragmented their DNA in vivo, but it clearly shows that they were in a preapoptotic state. Similarly, when unsorted splenocytes, PEC, or lung leukocytes were cultured for 5 hr, much higher proportions of the splenocytes were stained by TUNEL assay (Figure 4B). Hence, the organ-dependent differences were revealed by a reliable indicator of apoptosis shortly after placement in culture.

Organ-Dependent Differences in Susceptibility to AICD

Spleen T cells of LCMV-infected mice become very susceptible to AICD stimulated by triggering the TCR with anti-CD3 antibody (Razvi and Welsh, 1993). Leukocytes from spleen, representing the lymphoid organs, and peritoneal cavity, representing the peripheral organs, were therefore placed into culture and stimulated with anti-CD3 and IL-2. Thy1.1$^+$ mice reconstituted with Thy1.2$^+$ GP33-specific transgenic T cells were used, such that the percentage of Annexin-V and TUNEL staining and the number of antigen-specific transgenic Thy1.2$^+$ T cells could be enumerated by FACS. Figure 5A shows the effect of anti-CD3 stimulation on a naive transgenic T cell population. To simulate a normal control environment, spleen leukocytes from naive TCR-transgenic Thy 1.2$^+$ mice were mixed 1:3 with spleen leukocytes from uninfected control Thy1.1$^+$ mice, and PEC from transgenic mice were mixed 1:3 with PEC from control mice. A 40 hr treatment with anti-CD3 caused little change in number (No:) in either the naive spleen or PEC populations compared to unstimulated cells and did not induce Annexin-V^{bright} staining on either population, indicating that apoptosis was not being induced in the naive populations at this time point.

The effects of a 40 hr anti-CD3 treatment were quite different in leukocyte populations taken from day 9 LCMV-infected mice (Figure 5B). Anti-CD3 treatment of transgenic T cells from LCMV-infected spleens caused a >80% reduction in their number in comparison to unstimulated controls, and the great proportion (>60%) of the residual cells stained with Annexin-V, indicative of high levels of AICD. In marked contrast, anti-CD3 induced a slight increase in the number of transgenic T cells from the LCMV-infected peritoneal cavity, and most of the residual cells (>60%) were defined as Annexin-V$^-$. These results were supported by TUNEL stains shown from the same experiment in Figure 5B. Analyses of other lymph nodes gave results similar to spleen, and analysis of T cells from fat pads and lung gave results similar to the PEC (data not shown). This experiment argues that virus-specific splenic CD8 T cells devoid of the splenic architecture are much more susceptible to AICD than are peritoneal T cells.

We next questioned whether the sensitivity to apoptosis was an intrinsic property of the T cell or whether the leukocyte environment controlled this process. Therefore, PEC from LCMV-infected mice containing the transgenic T cells were mixed with 9-fold excess splenocytes from LCMV-infected mice not containing the transgenic T cells, and spleen cells from LCMV-infected mice containing the transgenic T cells were mixed with 9-fold excess PEC from LCMV-infected mice not con-

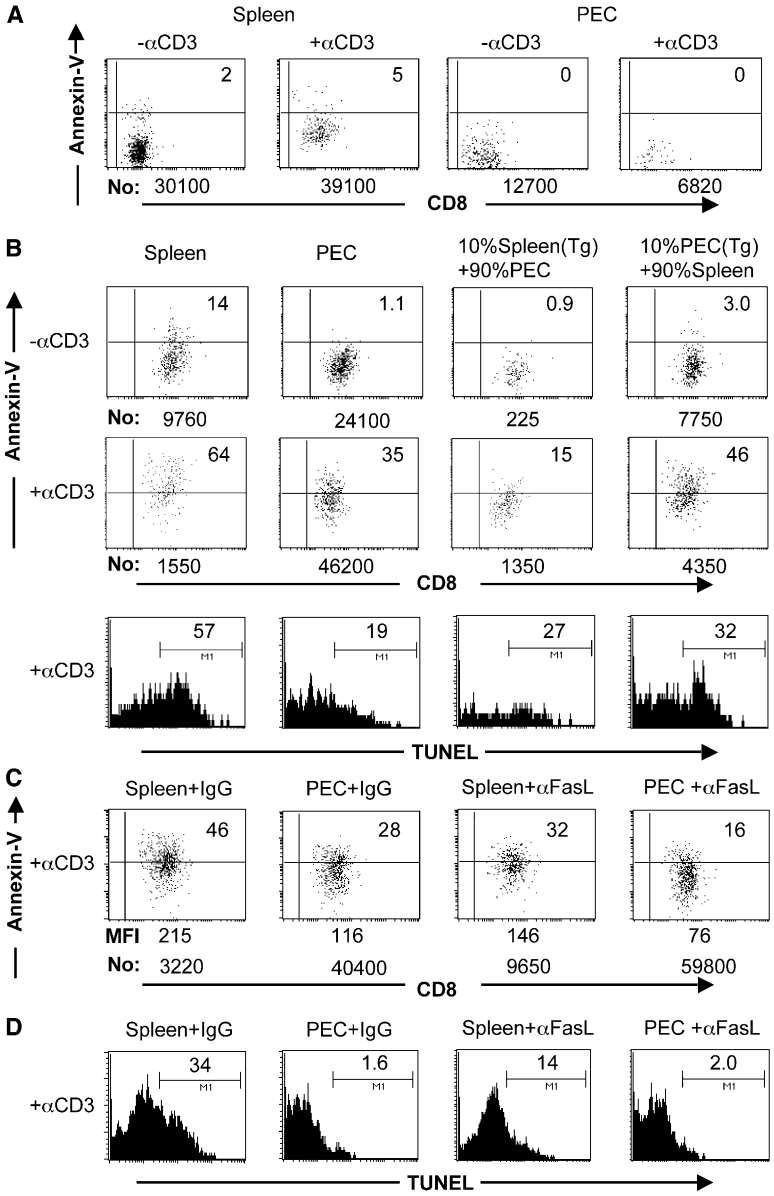

Figure 5. Resistance of PEC but Not Spleen T Cells to AICD and Influence of the Leukocyte Environments

Data were gated on the Thy1.2$^+$ transgenic T cells, as in Figure 3.

(A) Naive T cells. Spleen leukocytes or PEC from the Thy1.2$^+$ TCR-transgenic uninfected mice were mixed 1:3, respectively, with spleen leukocytes or PEC from uninfected Thy1.1$^+$ mice, to simulate a normal environment in which the transgenic T cells could be traced after stimulation in vitro. They were then cultured with or without anti-CD3, all in the presence of IL-2 for 40 hr. The panels show the Annexin-V versus anti-CD8 staining of the transgenic T cells.

(B–D) In vivo-activated T cells. Nine days after LCMV infection, spleen leukocytes and PEC from Thy1.1$^+$ mice reconstituted with Thy1.2$^+$ transgenic T cells were stimulated with anti-CD3 and IL-2 as in group (A). (B) The panels on the left show results from splenocytes or PEC cultured separately. The panels on the right show experiments where splenocytes from a day 9 LCMV-infected transgenic T cell-containing mouse were mixed 1:9 with PEC from a day 9 LCMV-infected Thy1.1$^+$ mouse not reconstituted with transgenic T cells, or else PEC from a transgenic-T cell-containing mouse mixed 1:9 with splenocytes from a day 9 LCMV-infected mouse not containing transgenic T cells (C and D). LCMV-infected spleen and PEC cells were incubated with anti-CD3 and with IgG control or anti-FasL for 40 hr and stained with anti-CD8, anti-Thy1.2, and Annexin-V (C) or, in a separate experiment, TUNEL stain (D). The number on each panel indicates the % of Annexin-V- or TUNEL-staining cells. All incubations were set up at 10^6 cells per well, and cells from replica wells were pooled for FACS analysis. Total viable cells were counted by trypan blue stain; the number under each panel represents the number of transgenic T cells remaining per well after 40 hr of stimulation. This was calculated by multiplying the total number of viable cells times the percentage of transgenic cells.

taining the transgenic T cells (Figure 5B). Putting the sensitive splenic T cells into the PEC environment resulted in a substantial increase (>5-fold) in their number after anti-CD3 treatment, and few of the cells stained with Annexin-V, consistent with the concept that Annexin-V reactivity is not simply a measure of T cell activation. Conversely, putting PEC T cells into the splenocyte environment caused a modest reduction in their number, and a high frequency of them stained with Annexin-V after the anti-CD3 treatment. This pattern was seen in three separate experiments, and the trend of reduced apoptosis of spleen cells in the PEC environment (57%→27%) and increased susceptibility of PEC T cells in the splenic environment (19%→32%) was also seen with the TUNEL stain (Figure 5B). Thus, the leukocyte environment influenced the sensitivity of the T cells to AICD. Additional experiments showed that spleen transgenic T cells were also protected from apoptosis by PEC depleted of T cells by anti-Thy1.1 and C′ (60%→14%).

AICD of LCMV-induced T cell populations depends on Fas/FasL interactions (Lohman et al., 1996). Here, either antibody to FasL or an isotype control was added into the 40 hr AICD assays. In three experiments, 10 μg/ml anti-FasL caused an average of a 2.5 ± 0.9-fold ($p = 0.03$) increase in numbers of the splenic transgenic T cells and an average of 41% ± 10% reduction in the % of Annexin-V$^+$ splenocytes ($p = 0.02$) and a 33% ± 3.8% reduction of MFI ($p = 0.04$) (one-tailed, paired T test). A representative experiment is shown in Figure 5C. A similar experiment using the TUNEL stain in Figure 5D shows a dramatic loss in TUNEL$^+$ spleen cells in the presence of anti-FasL; here, very few TUNEL$^+$ cells were in the PEC. These results confirm that the AICD observed

here, in contrast to the apoptosis associated with the silencing of the T cell response in vivo, was predominately mediated by Fas/FasL interactions.

Factors Contributing to the Tissue-Dependent Differences in Apoptosis

We questioned whether there were tissue-dependent differences in expression of genes known to regulate T cell apoptosis and first focused on the apoptotic regulators Bcl-2 and cFLIP, thought to be upregulated in memory T cell populations (Grayson et al., 2000; Inaba et al., 1999). We tested for Bcl-2 protein expression by immunofluorescence of transgenic T cells from different tissues and found no significant differences; the MFI in the transgenic T cells isolated from spleen, axial lymph node, PEC, and lung were 26, 29, 29, and 25, respectively. The MFI of an IgG isotype control in each organ was <6. Because cFLIP is transcriptionally controlled, we assessed its mRNA expression as part of a DNA microarray experiment (Inaba et al., 1999). Spleen and PEC transgenic T cells were sorted to at least 98% purity in three experiments, two at day 7 and one at day 9 p.i. The gene expression patterns of spleen and PEC T cells were compared using Affymetrix DNA microarray MG_U74Av2. The experiments revealed no significant differences in FLIP mRNA expression (signals at D7, D7 and D9: spleen-328, 430, 380; PEC-271, 367, 357). These experiments suggest that the reason for PEC T cell resistance to apoptosis is not because they are more memory-like than spleen T cells. Of the 141 apoptosis-related genes analyzed, 82 genes had a normalized signal intensity flagged as "present" in either the spleen or PEC in at least one of three experiments. Of these 82 genes, only granzyme A had a greater than 2.2-fold different normalized signal intensity between spleen and PEC. Granzyme A expression in the spleen T cells was 6, 3.6, and 13 times greater than in the PEC T cells (signals at D7, D7 and D9: spleen-12123, 7274, 9421; PEC-2034, 2016, 720).

The genechip analyses did not detect sufficiently strong signals of Fas mRNA to analyze, but differences in cell surface Fas protein expression were shown by immunofluorescence staining of LCMV-specific transgenic T cells isolated from the various tissues. Figure 6A shows immunofluorescent staining with mAb to Fas and FasL in freshly isolated leukocyte populations. The results indicated that antigen-specific T cells from the spleen and lymph node had nearly double the MFI for Fas as those from the PEC and lung. This observation was seen in three independent experiments. The freshly isolated spleen and lymph node T cells also had slightly higher staining for FasL, a result of questionable significance.

Expression of FasL on activated T cells tends to be transient, and optimal expression requires a recent stimulation through the TCR (Nagata and Golstein, 1995). We questioned whether in vitro stimulation with antigen would reveal significant tissue-dependent differences in Fas or FasL expression. Therefore, leukocytes isolated from day 9 LCMV-infected GP33-specific transgenic T cell-containing spleen or PEC were cultivated for 5 hr in the presence of control NP396 peptide, specific GP33 peptide, or anti-CD3 in order to stimulate the TCR. The differences in Fas expression between freshly isolated spleen and PEC T cells disappeared after 5 hr in culture, possibly due to death of the Fas-bearing cells, and these expression levels were virtually identical to those of naive T cells (data not shown). However, the responses of the residual T cells from the spleen and lymph node to stimulation were markedly different from those from the peripheral tissue. Stimulation of splenocytes with GP33 or anti-CD3 but not with the control NP396 peptide caused about a 2- to 2.5-fold enhancement in MFI for Fas in spleen transgenic T cells (Figure 6B) and axial lymph nodes (data not shown). In the PEC (Figure 6B) and lung (data not shown) transgenic T cells, this stimulation also caused an upregulation of Fas, but it was considerably less than that observed with the splenocytes (Figure 6B).

In vitro stimulation with GP33 or anti-CD3 but not NP396 similarly caused about a 2-fold (100%) increase in MFI for FasL in the transgenic T cells from the spleen (Figure 6B) and lymph node (data not shown). Increases in MFI for FasL in transgenic T cells in the PEC (Figure 6B) and lung (data not shown) were much less (about 30%; this low-level increase was seen in each of three experiments). Interestingly, when spleen transgenic T cells were placed into an environment of excess PEC, as described in Figure 5, their expression of FasL decreased greatly (e.g., FasL MFI in spleen $= 45$, in PEC $= 14$).

Addition of soluble FasL enhanced the Annexin-V reactivity of peritoneal transgenic T cells in 5 hr assays in the presence or absence of TCR stimulation. This indicates that some PEC T cells could be driven into apoptosis on receipt of a strong proapoptotic stimulus (Figure 6C), but even in the presence of FasL, the PEC were more resistant than spleen T cells. This result is likely influenced greatly by the reduced expression of Fas on the PEC T cells (Figure 6B), an interpretation supported by costaining cells with Annexin-V and antibody to Fas, showing that cells from either the spleen or PEC could undergo apoptosis if they expressed Fas. For example, after exposure to anti-CD3 and 100 ng FasL, day 9 spleen CD8 T cells defined as Annexin-V^+ (>200 MFI) had a Fas MFI of 233, and Annexin-V^- spleen T cells had an Fas MFI of 87; PEC CD8 T cells defined as Annexin-V^+ had a Fas MFI of 187, and Annexin-V^- had a Fas MFI of 97. Figure 6D shows the trend of high Fas and high Annexin-V costaining in a similar experiment using 50 ng/ml FasL.

Discussion

High frequencies of antigen-specific T cells accumulate in peripheral organs during infections and remain there as memory cells for prolonged time periods (Chen et al., 2001; Hogan et al., 2001; Masopust et al., 2001; Reinhardt et al., 2001). It has been suggested that T cells leave the spleen and lymph nodes and enter the periphery during the later stages of a T cell response and that the total loss in T cell number during the silencing phase of the response is proportionally greater in the lymphoid than in the peripheral tissues (Marshall et al., 2001; Masopust et al., 2001; Reinhardt et al., 2001). Memory

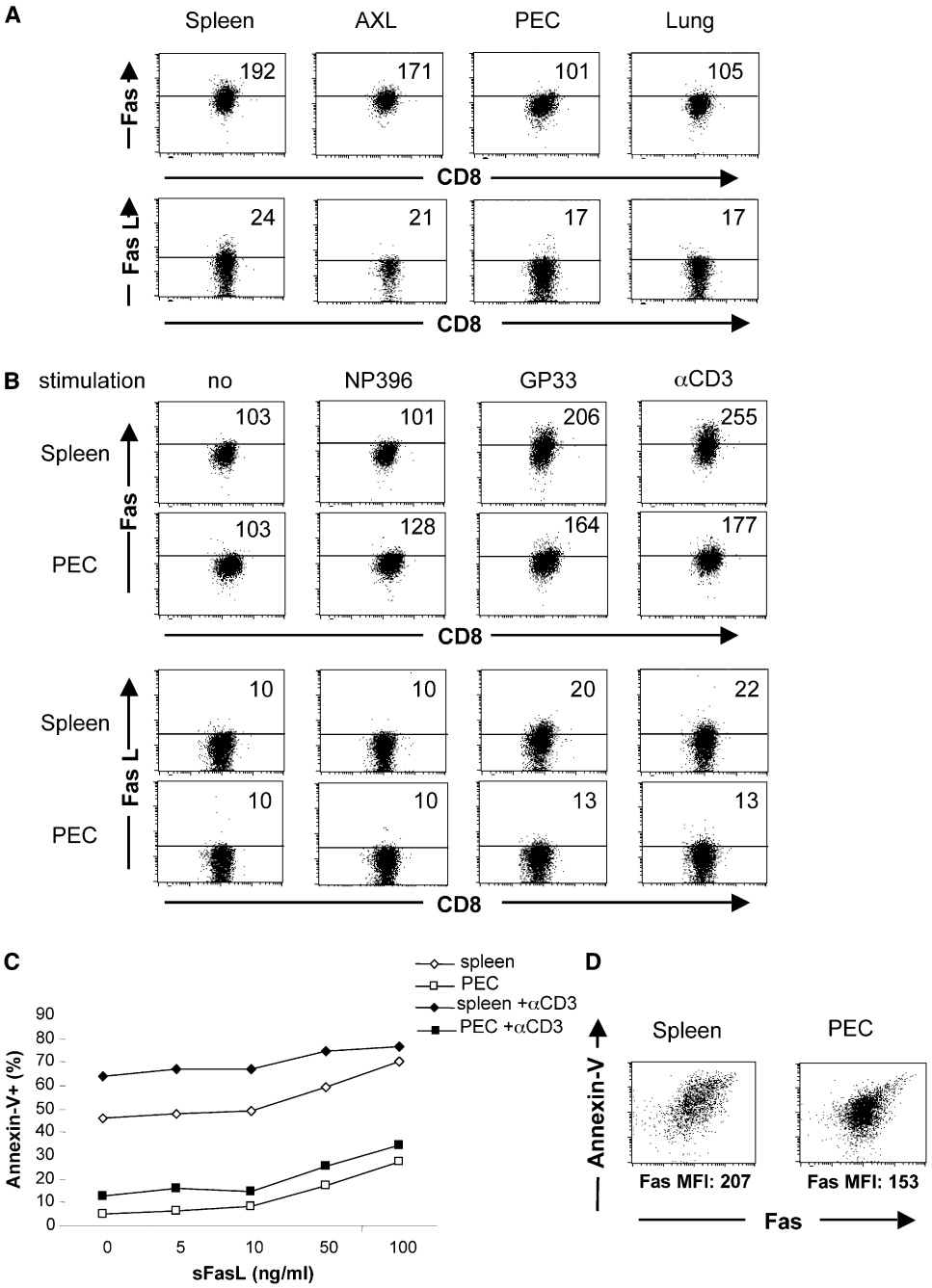

Figure 6. Fas and FasL Expression of LCMV-Specific Transgenic T Cells 8 Days after Infection and sFasL-Induced Apoptosis

(A) Fas and FasL staining of freshly isolated LCMV-specific transgenic T cells. (B) Fas and FasL staining of restimulated LCMV-specific transgenic T cells. Lymphocytes were isolated from LCMV-infected mice as described in Figure 3 and stained with mAb to CD8, Thy1.2, and Fas or FasL immediately (A) or were stimulated with peptide or anti-CD3 mAb for 5 hr and stained.

(B) Data were gated on $CD8^+$ $Thy1.2^+$ donor transgenic T cells. The numbers in the upper right quadrants of each panel represent the MFI of Fas or FasL staining of corresponding organ. The line is drawn directly above the unstimulated sample in order to highlight the increases seen after stimulation. Similar results were observed in mice from 10 or 12 days after infection.

(C) % of Annexin-V staining of day 9 LCMV-infected spleen (open diamond) and PEC (filled square) transgenic T cells incubated with sFasL for 5 hr.

(D) Annexin-V and Fas staining of CD8 T cells from anti-CD3-stimulated spleen and PEC with 50 ng/ml sFasL for 16 hr.

T cells in the spleen and lymph nodes undergo homeostatic division to maintain their frequencies (Harris et al., 2002; Razvi et al., 1995b; Tough and Sprent, 1994; Zimmermann et al., 1996a), but T cells in the periphery have reduced cell division and slowly decline in number over a period of several months (Harris et al., 2002). Peripheral T cells, though not rapidly dividing, are at relatively high activation states, as defined by cytolytic

activity and activation antigen expression, and this may enable them to be uniquely suited for protective immunity (Chen et al., 2001; Hogan et al., 2001; Masopust et al., 2001; Reinhardt et al., 2001). Under conditions where memory T cell frequencies in the spleen and lymph node are maintained, protective T cell immunity against a peripheral viral challenge can be lost if memory T cells in the periphery have declined in number (Hogan et al., 2001).

Our studies reveal additional dramatic differences between lymphoid and peripheral CD8 T cells and suggest mechanisms that explain the high frequencies in the periphery and why they may be uniquely suited for effectively engaging pathogens. First of all, a surprisingly high proportion of antigen-specific T cells in the spleen and lymph nodes display a preapoptotic phenotype, as shown by Annexin-V binding, but those T cells residing in the periphery do not. We cannot be sure that all of these preapoptotic cells complete the apoptotic process in vivo, but they rapidly fragment their DNA when placed in culture (Figure 4A). Second, whereas the activated T cells in the spleen undergo AICD and fail to proliferate on TCR ligation in vitro (Razvi and Welsh, 1993), those in the periphery have increased survival and resistance to AICD (Figure 5). This resistance to AICD by peripheral T cells is associated with their poor expression of the proapoptotic molecule FasL after signaling through their TCR and to their low expression of its signaling receptor, Fas (Figure 6). Given that T cell apoptosis may be influenced by FasL-expressing T cells in their environment (Nagata and Golstein, 1995), it is not surprising that susceptibility to AICD was conferred on peripheral T cells placed into an environment of excess FasL-bearing cells from lymphoid organs (Figure 5). Conversely, when spleen transgenic T cells were put into a peripheral leukocyte environment, they resisted apoptosis and downregulated FasL expression.

Immunofluorescence data also showed that lymphoid organ T cells expressed higher levels of Fas and FasL than did peripheral tissue T cells, consistent with their enhanced sensitivity to AICD. No other tested activation markers (1B11(CD43), CD44, CD69, CD122, Ly6C) were expressed more highly on lymphoid than peripheral T cells. Of note is that we found no differences in freshly isolated cells in expression of Bcl-2, an antiapoptotic protein reported to be expressed higher in memory than in activated LCMV-specific CD8 T cells (Grayson et al., 2000). Our earlier studies had found no significant role for Bcl-2 in the overall silencing of the CD8 T cell response in the spleen or in the sensitivity of LCMV-induced splenic T cells to AICD (Razvi et al., 1995a).

An important question is why T cells in the peripheral tissues have different expression of Fas and FasL and different apoptotic properties than those in the spleen and lymph nodes. Antigen-presenting cells delivering signals to the T cells may differ in these organs, as CD8 T cells in peripheral tissue may be stimulated mostly by parenchymal cells, macrophages, and/or immature dendritic cells, whereas those in the lymphoid organs may be stimulated primarily by mature dendritic cells. Expression of Fas and FasL on lymphocytes can be altered by integrins and cytokines, such as $\alpha 1\beta 2$ integrins, IL-2, IL-6, INFα, and TGFβ1, leading to a modified susceptibility to AICD (Aoudjit and Vuori, 2000; Ayroldi et al., 1998; Genestier et al., 1999; Kaser et al., 1999; Van Parijs et al., 1999). Leukocytes from peripheral organs have high percentages of activated macrophages, which produce high levels of TGFβ, shown to inhibit FasL expression (Assoian et al., 1987; Genestier et al., 1999). In three experiments adding either antibody to TGFβ or an isotype control antibody into 40 hr AICD assays, 10 μg/ml of anti-TGFβ caused an average of 10% $\pm$ 5% increase in the MFI of Annexin-V splenocytes ($p = 0.04$) and a 37% $\pm$ 11% increase in the MFI in PEC ($p = 0.03$). TGFβ may, then, be one of perhaps several factors protecting peripheral T cells from AICD.

The spontaneous apoptosis during the silencing of the T cell response occurs by a different mechanism than the TCR-induced AICD. Silencing of the immune response occurs normally in mice bearing mutations in Fas or FasL (Lohman et al., 1996; Razvi et al., 1995a; Zimmermann et al., 1996b), and we show here that T cells from LCMV-infected lpr and gld mice have Annexin-V staining patterns similar to those in normal infected mice. The heightened expression of Fas may herald other genes contributing to a proapoptotic intracellular environment. Of interest, however, is that few changes in apoptotic gene expression were seen from freshly isolated T cells, with the exception of granzyme A, which was considerably higher in spleen T cells. Granzyme A is a protease thought to be involved mostly in T cell-dependent cytotoxicity rather than T cell apoptosis (Jenne and Tschopp, 1988). Future work should determine what role, if any, it plays in the silencing of the T cell response or in augmenting the sensitivity of these cells to AICD.

The tissue-dependent differences in preapoptotic cells were not confined to a unique time period, but occurred at 7, 9, and 12 days after acute LCMV infection and 2 and 4 months later in the memory state. We were surprised at the relatively high frequencies of Annexin-V-reactive memory T cells 2–4 months postinfection, as these frequencies are higher than that which would be predicted of the number of cells in cycle, as shown by BrdU studies (Zimmermann et al., 1996a). It is interesting to note, however, that memory cells in lymphoid organs are constantly undergoing homeostatic division without an increase in number, indicating that an apoptotic event must offset each cell division.

A differential propensity to apoptosis may play a role in the overall maintenance of antigen-specific T cells in lymphoid and nonlymphoid tissue. If effective memory to peripheral challenge does wane with time and corresponds with the decline in frequency in peripheral memory cells (Hogan et al., 2001), then it would serve the host to preserve the functional integrity of such cells, to maintain their viability, and to have them respond productively rather than apoptotically to antigen stimulation. We show here that reduced sensitivity of peripheral T cells to apoptosis on exposure to ligand provides a mechanism for maintaining their numbers and their functional integrity.

Experimental Procedures

Mice

C57BL/6J and B6.PLThy1^a/C (Thy1.1) male mice were purchased from the Jackson Laboratory (Bar Harbor, ME). B6.SJL (Ly5.1) male mice were from Taconic labs (Germantown, NY). Mice were used

at 6–12 weeks of age. P-14 TCR transgenic mice that express a transgenic TCR specific to the LCMV-encoded class I MHC-presented peptide GP_{33-41} (Pircher et al., 1989) were purchased from Jackson Laboratories and were backcrossed onto $RAG^{-/-}$ C57BL/6 mice (Thy 1.2 Ly5.2) by Dr. Joseph Maciaszek at UMMS.

Virus

Mice were inoculated intraperitoneally (i.p.) with 0.1 ml of 4×10^4 plaque-forming units (PFU) of the Armstrong strain of LCMV diluted in PBS (Razvi and Welsh, 1993).

Preparation of Leukocytes

Mice were anesthetized by an i.p. injection of Nembutal (Abbott laboratories, IL). Leukocytes from the PEC were collected by lavaging with 10 ml cold RPMI 1640 medium (GIBCO, Gaithersburg, MD). The lung vascular bed was flushed with 10 ml chilled Hank's balanced salt solution (HBSS, GIBCO) introduced via cannulation of the right ventricle of the heart. Blood from liver was flushed by injecting 5 ml of RPMI through the portal vein. Single-cell suspensions from spleens and lymph nodes were made by grinding the organs between glass microscope slides. Fat pads and lungs were minced and incubated for 45–60 min at 37°C in 0.5 mg/ml type II collagenase (Sigma-Aldrich, St. Louis, MO) and 100 U/ml type I DNase (Sigma-Aldrich). Lung leukocytes were collected by a Lympholyte-M (Cedarlane Labs, Hornby, Canada) density gradient (Chen et al., 2001). Leukocytes from the liver were isolated by crushing the liver in a tissue grinder, incubating with enzyme solution, and collecting the leukocyte layer from a metrizamide (Sigma-Aldrich) density gradient (Daniels et al., 2001). Contaminating erythrocytes were removed from the leukocyte preparations by treatment with 0.84% NH_4Cl.

$H2D^b$- and $H2K^b$-IgG1 MHC Dimer- and Annexin-V-Staining of Leukocytes

D^b-IgG1 dimers (Greten et al., 1998; Selin et al., 1999) were used with the LCMV immunodominant peptides $NP_{396-404}$ (FQPQNGQFI) (Gairin et al., 1995) and GP_{33-41} (AVYNFATC) (Whitton et al., 1988). K^b-IgG1 dimers (Pharmingen) were used with the subdominant $NP_{205-212}$ (YTVKYPNL) peptide (van der Most et al., 1998). The dimers were incubated with 0.15 μg β2-microglobulin and 800-fold molar excess peptide for 7 days at 4°C. For staining, 1 μg of peptide-charged dimer was incubated with 10^6 leukocytes for 1.5 hr at 4°C. The cells were then incubated with biotinylated anti-mouse IgG1 and PerCP-anti-CD8, and then with PE-streptavidin (Pharmingen), all done at 4°C. The cells were then washed and incubated in Annexin-V binding buffer with FITC-Annexin-V (Pharmingen) at a 1:20 dilution for 15 min at room temperature. The cells were washed, resuspended in Annexin-V binding buffer, and analyzed by flow cytometry immediately.

$H2D^b$ MHC Tetramer Staining and Sorting

10^6 leukocytes were incubated with PE-NP396-specific $H2D^b$ tetramer (Murali-Krishna et al., 1998; Mylin et al., 2000) and FITC-anti-CD8 (Pharmingen) for 1 hr at 4°C. After staining, the cells were sorted by flow cytometry using a FACSTAR sorter.

CDR3 Length Spectratyping Analysis

CDR3 length "spectratype" analysis was done as described (Lin and Welsh, 1998; Pannetier et al., 1993). In brief, RNA samples were amplified with primers for Cβ and for Vβ8.1, using a GeneAmp RNA kit (Perkin-Elmer Corp., Branchburg, NJ). The PCR products were subjected to five cycles of runoff reaction with 6 fluorophore-labeled Jβ primers (Jβ 1.1, 1.2, 1.3, 1.4, 1.5, and 2.1). The runoff products were loaded onto a 4.75% acrylamide sequencing gel and analyzed on an automated DNA sequencer using GeneScan software (Applied Biosystems, Foster City, CA).

Adoptive Transfer Studies

Two $\times 10^6$ leukocytes from spleen and lymph nodes of P14 LCMV-specific TCR transgenic $RAG^{-/-}$ Thy1.2 mice were injected intravenously via the retro-orbital sinus into B6.PL Thy1a/C (Thy1.1) (or B6.SJL (Ly5.1) for one D 7 Genechip experiment) mice. The mice were inoculated with LCMV 1 to 3 days thereafter. At 8 to 12 days p.i., leukocytes were isolated from different organs, and donor cells were identified by flow cytometry using anti-Thy1.2 mAb (Pharmingen). The proportion of transgenic T cells varied in the leukocyte population from different tissues, with the ranges at day 9 postinfection being 3%–5% for spleen, 1%–2% for LN, 30%–40% for PEC, 10%–20% for lung, and 30%–40% for fat pad.

Induction of AICD In Vitro and TUNEL Staining

Leukocytes were stimulated with mAb to CD3 (145-2C11; Pharmingen) in vitro in 48-well tissue culture plates coated with 2 μg purified anti-CD3 mAb overnight at 4°C. Other wells were sham-treated. To each well were then added freshly isolated 10^6 leukocytes along with 0.02 μg human recombinant interleukin-2 (IL-2) (Pharmingen) in 1 ml MLC-RPMI medium (Lohman and Welsh, 1998). These cells were then cultured for 40 hr at 37°C and analyzed by flow cytometry for antigen specificity, cell number, and apoptotic properties. Anti-FasL (clone MFL3) or an IgG isotype control (10 μg/ml) (eBioscience, San Diego, CA) was added to spleen or PEC leukocytes. Cells were incubated with anti-CD3 for 40 hr and stained with antibodies. Sensitivity to FasL-induced apoptosis was monitored by adding soluble FasL and a crosslinking enhancer Ig to cells for 5 hr at 37°C, according to manufacturer's instructions (Alexis, San Diego, CA). TUNEL staining was applied with a terminal transferase kit (Roche, Indianapolis, IN) according to the manufacturer's instructions.

Immunofluorescent Staining with Antibodies to Fas, FasL, and Bcl-2

Leukocytes were stained with mAb to PerCP-CD8, APC-Thy1.2, and Biotin-Fas (clone Jo2) or FasL (clone Kay-10) (Pharmingen) for 30 min at 4°C. In some cases, cells were stained intracytoplasmically with a PE-Bcl-2 mAb (clone 3F11) or its IgG isotype control (clone A19-3) with Cytofix/Cytoperm Kit (Pharmingen), according to the manufacturer's instructions. In some cases, leukocytes from LCMV-infected mice were first stimulated with 5 μM synthetic GP_{33-41} or $NP_{396-404}$ peptide or 2 μg anti-CD3 mAb for 5 hr at 37°C before staining with mAbs to Fas and FasL.

Gene Microarray Analysis

Affymetrix, Inc. (Santa Clara, CA) gene array analyses were done according to the manufacturer's procedures manual. Total RNA was isolated from cells using RNeasy Mini Kit (QIAGEN). cDNA was synthesized using SuperScript DS cDNA synthesis kit (Invitrogen, Life Technologies) with T7-$(dT)_{24}$ primer (Genset Corp). The biotin-labeled cRNA was transcribed and labeled using RNA transcript labeling kit (Enzo Diagnostics, Inc, Farmingdale, NY). Labeled cRNA was fragmented and hybridized to MG-U74Av2 microarray chips and expression analysis was done with Microarray Suite 5.0. "Apoptosis-related genes" were analyzed by GeneSpring 5.0 (Silicon Genetics, Redwood City, CA).

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References

Ahmed, R., and Gray, D. (1996). Immunological memory and protective immunity: understanding their relation. Science *272*, 54–60.

Aoudjit, F., and Vuori, K. (2000). Engagement of the alpha2beta1

integrin inhibits Fas ligand expression and activation-induced cell death in T cells in a focal adhesion kinase-dependent manner. Blood *95*, 2044–2051.

Assoian, R.K., Fleurdelys, B.E., Stevenson, H.C., Miller, P.J., Madtes, D.K., Raines, E.W., Ross, R., and Sporn, M.B. (1987). Expression and secretion of type beta transforming growth factor by activated human macrophages. Proc. Natl. Acad. Sci. USA *84*, 6020–6024.

Ayroldi, E., Zollo, O., Cannarile, L., D'Adimo, F., Grohmann, U., Delfino, D.V., and Riccardi, C. (1998). Interleukin-6 (IL-6) prevents activation-induced cell death: IL-2-independent inhibition of Fas/fasL expression and cell death. Blood *92*, 4212–4219.

Broome, H.E., Dargan, C.M., Krajewski, S., and Reed, J.C. (1995). Expression of Bcl-2, Bcl-x, and Bax after T cell activation and IL-2 withdrawal. J. Immunol. *155*, 2311–2317.

Chen, H.D., Fraire, A.E., Joris, I., Brehm, M.A., Welsh, R.M., and Selin, L.K. (2001). Memory CD8+ T cells in heterologous antiviral immunity and immunopathology in the lung. Nat. Immunol. *2*, 1067–1076.

Chen, W., Anton, L.C., Bennink, J.R., and Yewdell, J.W. (2000). Dissecting the multifactorial causes of immunodominance in class I-restricted T cell responses to viruses. Immunity *12*, 83–93.

Daniels, K.A., Devora, G., Lai, W.C., O'Donnell, C.L., Bennett, M., and Welsh, R.M. (2001). Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H. J. Exp. Med. *194*, 29–44.

Deng, G., and Podack, E.R. (1993). Suppression of apoptosis in a cytotoxic T-cell line by interleukin 2-mediated gene transcription and deregulated expression of the protooncogene bcl-2. Proc. Natl. Acad. Sci. USA *90*, 2189–2193.

Gairin, J.E., Mazarguil, H., Hudrisier, D., and Oldstone, M.B. (1995). Optimal lymphocytic choriomeningitis virus sequences restricted by H-2Db major histocompatibility complex class I molecules and presented to cytotoxic T lymphocytes. J. Virol. *69*, 2297–2305.

Genestier, L., Kasibhatla, S., Brunner, T., and Green, D.R. (1999). Transforming growth factor beta1 inhibits Fas ligand expression and subsequent activation-induced cell death in T cells via downregulation of c-Myc. J. Exp. Med. *189*, 231–239.

Grayson, J.M., Zajac, A.J., Altman, J.D., and Ahmed, R. (2000). Cutting edge: increased expression of Bcl-2 in antigen-specific memory CD8+ T cells. J. Immunol. *164*, 3950–3954.

Greten, T.F., Slansky, J.E., Kubota, R., Soldan, S.S., Jaffee, E.M., Leist, T.P., Pardoll, D.M., Jacobson, S., and Schneck, J.P. (1998). Direct visualization of antigen-specific T cells: HTLV-1 Tax11-19-specific CD8(+) T cells are activated in peripheral blood and accumulate in cerebrospinal fluid from HAM/TSP patients. Proc. Natl. Acad. Sci. USA *95*, 7568–7573.

Harris, N.L., Watt, V., Ronchese, F., and Le Gros, G. (2002). Differential T cell function and fate in lymph node and nonlymphoid tissues. J. Exp. Med. *195*, 317–326.

Hogan, R.J., Usherwood, E.J., Zhong, W., Roberts, A.A., Dutton, R.W., Harmsen, A.G., and Woodland, D.L. (2001). Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections. J. Immunol. *166*, 1813–1822.

Huang, L., Soldevila, G., Leeker, M., Flavell, R., and Crispe, I.N. (1994). The liver eliminates T cells undergoing antigen-triggered apoptosis in vivo. Immunity *1*, 741–749.

Inaba, M., Kurasawa, K., Mamura, M., Kumano, K., Saito, Y., and Iwamoto, I. (1999). Primed T cells are more resistant to Fas-mediated activation-induced cell death than naive T cells. J. Immunol. *163*, 1315–1320.

Jenne, D.E., and Tschopp, J. (1988). Granzymes, a family of serine proteases released from granules of cytolytic T lymphocytes upon T cell receptor stimulation. Immunol. Rev. *103*, 53–71.

Kaser, A., Nagata, S., and Tilg, H. (1999). Interferon alpha augments activation-induced T cell death by upregulation of Fas (CD95/APO-1) and Fas ligand expression. Cytokine *11*, 736–743.

Lenardo, M., Chan, K.M., Hornung, F., McFarland, H., Siegel, R., Wang, J., and Zheng, L. (1999). Mature T lymphocyte apoptosis–immune regulation in a dynamic and unpredictable antigenic environment. Annu. Rev. Immunol. *17*, 221–253.

Lin, M.Y., and Welsh, R.M. (1998). Stability and diversity of T cell receptor repertoire usage during lymphocytic choriomeningitis virus infection of mice. J. Exp. Med. *188*, 1993–2005.

Liu, Z.X., Govindarajan, S., Okamoto, S., and Dennert, G. (2001). Fas-mediated apoptosis causes elimination of virus-specific cytotoxic T cells in the virus-infected liver. J. Immunol. *166*, 3035–3041.

Lohman, B.L., and Welsh, R.M. (1998). Apoptotic regulation of T cells and absence of immune deficiency in virus-infected gamma interferon receptor knockout mice. J. Virol. *72*, 7815–7821.

Lohman, B.L., Razvi, E.S., and Welsh, R.M. (1996). T-lymphocyte downregulation after acute viral infection is not dependent on CD95 (Fas) receptor-ligand interactions. J. Virol. *70*, 8199–8203.

Mackay, C.R., Marston, W.L., Dudler, L., Spertini, O., Tedder, T.F., and Hein, W.R. (1992). Tissue-specific migration pathways by phenotypically distinct subpopulations of memory T cells. Eur. J. Immunol. *22*, 887–895.

Marshall, D.R., Turner, S.J., Belz, G.T., Wingo, S., Andreansky, S., Sangster, M.Y., Riberdy, J.M., Liu, T., Tan, M., and Doherty, P.C. (2001). Measuring the diaspora for virus-specific CD8+ T cells. Proc. Natl. Acad. Sci. USA *98*, 6313–6318.

Masopust, D., Vezys, V., Marzo, A.L., and Lefrancois, L. (2001). Preferential localization of effector memory cells in nonlymphoid tissue. Science *291*, 2413–2417.

Mullbacher, A., and Flynn, K. (1996). Aspects of cytotoxic T cell memory. Immunol. Rev. *150*, 113–127.

Murali-Krishna, K., Altman, J.D., Suresh, M., Sourdive, D.J., Zajac, A.J., Miller, J.D., Slansky, J., and Ahmed, R. (1998). Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity *8*, 177–187.

Mylin, L.M., Schell, T.D., Roberts, D., Epler, M., Boesteanu, A., Collins, E.J., Frelinger, J.A., Joyce, S., and Tevethia, S.S. (2000). Quantitation of CD8(+) T-lymphocyte responses to multiple epitopes from simian virus 40 (SV40) large T antigen in C57BL/6 mice immunized with SV40, SV40 T-antigen-transformed cells, or vaccinia virus recombinants expressing full-length T antigen or epitope minigenes. J. Virol. *74*, 6922–6934.

Nagata, S., and Golstein, P. (1995). The Fas death factor. Science *267*, 1449–1456.

Pannetier, C., Cochet, M., Darche, S., Casrouge, A., Zoller, M., and Kourilsky, P. (1993). The sizes of the CDR3 hypervariable regions of the murine T-cell receptor beta chains vary as a function of the recombined germ-line segments. Proc. Natl. Acad. Sci. USA *90*, 4319–4323.

Petschner, F., Zimmerman, C., Strasser, A., Grillot, D., Nunez, G., and Pircher, H. (1998). Constitutive expression of Bcl-xL or Bcl-2 prevents peptide antigen-induced T cell deletion but does not influence T cell homeostasis after a viral infection. Eur. J. Immunol. *28*, 560–569.

Pircher, H., Burki, K., Lang, R., Hengartner, H., and Zinkernagel, R.M. (1989). Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. Nature *342*, 559–561.

Razvi, E.S., and Welsh, R.M. (1993). Programmed cell death of T lymphocytes during acute viral infection: a mechanism for virus-induced immune deficiency. J. Virol. *67*, 5754–5765.

Razvi, E.S., Jiang, Z., Woda, B.A., and Welsh, R.M. (1995a). Lymphocyte apoptosis during the silencing of the immune response to acute viral infections in normal, lpr, and Bcl-2-transgenic mice. Am. J. Pathol. *147*, 79–91.

Razvi, E.S., Welsh, R.M., and McFarland, H.I. (1995b). In vivo state of antiviral CTL precursors. Characterization of a cycling cell population containing CTL precursors in immune mice. J. Immunol. *154*, 620–632.

Reich, A., Korner, H., Sedgwick, J.D., and Pircher, H. (2000). Immune down-regulation and peripheral deletion of CD8 T cells does not require TNF receptor-ligand interactions nor CD95 (Fas, APO-1). Eur. J. Immunol. *30*, 678–682.

Reinhardt, R.L., Khoruts, A., Merica, R., Zell, T., and Jenkins, M.K.

(2001). Visualizing the generation of memory CD4 T cells in the whole body. Nature *410*, 101–105.

Russell, J.H., White, C.L., Loh, D.Y., and Meleedy-Rey, P. (1991). Receptor-stimulated death pathway is opened by antigen in mature T cells. Proc. Natl. Acad. Sci. USA *88*, 2151–2155.

Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature *401*, 708–712.

Selin, L.K., Lin, M.Y., Kraemer, K.A., Pardoll, D.M., Schneck, J.P., Varga, S.M., Santolucito, P.A., Pinto, A.K., and Welsh, R.M. (1999). Attrition of T cell memory: selective loss of LCMV epitope-specific memory CD8 T cells following infections with heterologous viruses. Immunity *11*, 733–742.

Tough, D.F., and Sprent, J. (1994). Turnover of naive- and memory-phenotype T cells. J. Exp. Med. *179*, 1127–1135.

van der Most, R.G., Murali-Krishna, K., Whitton, J.L., Oseroff, C., Alexander, J., Southwood, S., Sidney, J., Chesnut, R.W., Sette, A., and Ahmed, R. (1998). Identification of Db- and Kb-restricted subdominant cytotoxic T-cell responses in lymphocytic choriomeningitis virus-infected mice. Virology *240*, 158–167.

van Engeland, M., Nieland, L.J., Ramaekers, F.C., Schutte, B., and Reutelingsperger, C.P. (1998). Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry *31*, 1–9.

Van Parijs, L., Refaeli, Y., Lord, J.D., Nelson, B.H., Abbas, A.K., and Baltimore, D. (1999). Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death. Immunity *11*, 281–288.

Whitton, J.L., Gebhard, J.R., Lewicki, H., Tishon, A., and Oldstone, M.B. (1988). Molecular definition of a major cytotoxic T-lymphocyte epitope in the glycoprotein of lymphocytic choriomeningitis virus. J. Virol. *62*, 687–695.

Zimmermann, C., Brduscha-Riem, K., Blaser, C., Zinkernagel, R.M., and Pircher, H. (1996a). Visualization, characterization, and turnover of CD8+ memory T cells in virus-infected hosts. J. Exp. Med. *183*, 1367–1375.

Zimmermann, C., Rawiel, M., Blaser, C., Kaufmann, M., and Pircher, H. (1996b). Homeostatic regulation of CD8+ T cells after antigen challenge in the absence of Fas (CD95). Eur. J. Immunol. *26*, 2903–2910.

Zinkernagel, R.M., Bachmann, M.F., Kundig, T.M., Oehen, S., Pirchet, H., and Hengartner, H. (1996). On immunological memory. Annu. Rev. Immunol. *14*, 333–367.